Zoosyst. Evol. 100 (2) 2024, 721–738 | DOI 10.3897/zse.100.118051

PENSOFT.

für Natur MUSEUM FÜR NATURKUNDE BERLIN

Two new species of groundwater-inhabiting amphipods belonging to the genus *Niphargus* (Arthropoda, Crustacea), from Iran

Seyyed Ahmad Mirghaffari[1], Somayeh Esmaeili-Rineh[1]

1 *Department of Biology, Faculty of Science, Razi University, Baghabrisham 6714967346, Kermanshah, Iran*

https://zoobank.org/7982E5C8-B398-4249-97D8-C9DBB82752A1

Corresponding author: Somayeh Esmaeili-Rineh (sesmaeili@razi.ac.ir)

Academic editor: Luiz F. Andrade ♦ **Received** 31 December 2023 ♦ **Accepted** 22 March 2024 ♦ **Published** 5 June 2024

Abstract

In Iran, the Elburz and Zagros Mountain ranges include substantial karst regions housing numerous aquifers and groundwater resources. *Niphargus* Schiødte, 1849, a diverse subterranean amphipod genus, inhabits Western Palearctic groundwater environments, with Iran marking the eastern limit of its distribution. This study examined specimens collected from springs along the Elburz and Zagros Mountains, revealing two distinct taxonomic units through a combination of morphological observations and molecular analyses, utilizing COI and 28S rDNA genes. *N. elburzensis* **sp. nov.** is characterized by produced epimeral plates I to III; a telson lobe with five distal, two lateral, and one mesial spine each, a rectangular shape of gnathopod II propodi with two L-setae on palmar corner and maxilla I outer plate spines with 2-2-1-1-3-0-1 denticles. *N. zagrosensis* **sp. nov.** is distinguished by a triangular shape gnathopod II propodi, pereopod VI longer than pereopod VII, maxilliped outer plate less than half of palp article 2, and uropod III distal article exceeding 80% of the proximal article. Pairwise genetic distances between *N. elburzensis* **sp. nov.** and other species ranged from 10.70% (*N. fiseri*) to 23.48% (*N. daniali*) for COI gene and 1.56% (*N. urmiensis*) to 10.98% (*N. daniali*) for 28S gene. Also, *N. zagrosensis* **sp. nov.** exhibited COI gene distances from 5.73% (*N. alisadri*) to 20.66% (*N. daniali*) and from 0.13% (*N. alisadri*) to 11.36% (*N. daniali*) for 28S gene distances. Bayesian analysis suggests that the two newly discovered species are part of the expansive local Iranian clade. These species are supported phylogenetically by separate and independent lineages, as indicated by high bootstrapping values.

Key Words

Molecular analyses, morphological characters, novel species, subterranean amphipods

Introduction

Amphipod genus *Niphargus* encompasses around 500 known species, making it one of the most diverse taxa in subterranean freshwater ecosystems. These organisms inhabit caves, springs, and subterranean streams, with only a limited number of species observed in surface waters (Fišer et al. 2009; Copilaş-Ciocianu and Boros 2016; Morhun et al. 2022; Marin and Palatov 2023). The diversity of *Niphargus* species in subterranean freshwater ecosystems exhibits a declining trend as one moves from southern to northern parts of Europe (Väinölä et al. 2008).

The classification of the genus *Niphargus* is complex due to limited distribution, habitat inaccessibility, homoplasy, and complex morphological diversity within species (Ozkahya and Camur-Elipek 2015; Fišer et al. 2018; Stoch et al. 2020). The application of molecular techniques has significantly facilitated the identification of species boundaries, particularly in cases where morphological characteristics may be unclear (Mamaghani-Shishvan and Esmaeili-Rineh 2019; Weber and Weigand 2023). Sometimes, molecular analysis reveals the presence of multi-species while morphologically classified as a single species. Consequently, it is recommended to use a combination of morphological and molecular characteristics for species identification and delimitation within this genus (Balázs et al. 2023; Weber and Weigand 2023).

Copyright *Mirghaffari, S.A. & Esmaeili-Rineh, S.* This is an open access article distributed under the terms of the Creative Commons Attribution License (CC BY 4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Iran represents the eastern boundary of *Niphargus* range. Within this region, most of the species belong to a distinct clade that diverged from their European counterparts 11–9 million years ago (Esmaeili-Rineh et al. 2015; Borko et al. 2021). To date, more than 20 species of amphipods belonging to the genus *Niphargus* have been identified in Iran, and the greatest diversity is observed in subterranean aquifers within the Zagros Mountains region (Esmaeili-Rineh et al. 2015). Approximately 10% of Iran's land area is composed of karst formations, predominantly found within the Alborz and Zagros mountain ranges; it is likely that these regions contain numerous additional species, with only a limited number discovered so far.

The present study, conducted from 2021 to 2023, encompassed sampling in various karst regions in Iran, leading to the discovery of two new species of subterranean freshwater amphipods from the Alborz and Zagros mountain ranges.

Materials and methods

Sampling area

The materials examined in this study were collected using a handnet and subsequently preserved in 70% and 96% ethanol for morphological and molecular studies, respectively. The geographic distribution of these materials is illustrated in Fig. 1. First, six samples (three for each species) were examined for morphological studies and then, one appendage was removed from each specimen for DNA analyses and the rest of the individuals were mounted on slides in Euparal® medium.

The slides were studied using a Zeiss Primostar microscope and a LABOMED Lx500 stereomicroscope. Details were photographed using an Olympus LABOMED iVu7000 camera mounted on the stereomicroscope. Measurements and counts of morphological characters were conducted using the digitized photos and the computer program PROGRES CAPTURE PRO 2.7, referring to characters and landmarks defined by Fišer et al. (2009). Illustrations were prepared in ADOBE ILLUSTRATOR CS5, using photos as background images. All specimens were deposited in the Zoological Collection of Razi University (ZCRU).

Molecular and phylogenetic analyses

The total genomic DNA was extracted from a part of an animal using Tissue Kits (GenNet Bio™, Seoul, Korea) following the manufacturer's instructions. The modified primer pair LCO1490-JJ and HCO2198-JJ (Astrin and Stüben 2008) were used to amplify a fragment of the mitochondrial COI gene. A fragment of 28S ribosomal DNA (rDNA) was amplified and sequenced following Verovnik et al. (2005) and Zakšek et al. (2007), for the forward and reverse primer, respectively.

Each 25 µl PCR mix comprised of water, 12.5 µl of Master Mix kit (Sinaclon, Iran), 0.2 µl of each primer (10 µM), and 50–100 ng of genomic DNA template. An initial denaturation step at 94 °C for 3 minutes was followed by 36 cycles of 40 seconds at 94 °C, 40 seconds at 52.5 °C and 2 min at 65 °C with a final extension step of 8 minutes at 72 °C to amplify the COI gene. Cycling parameters for the 28S rDNA gene were as follows: initial denaturation of 94 °C for 7 minutes, 35 subsequent cycles

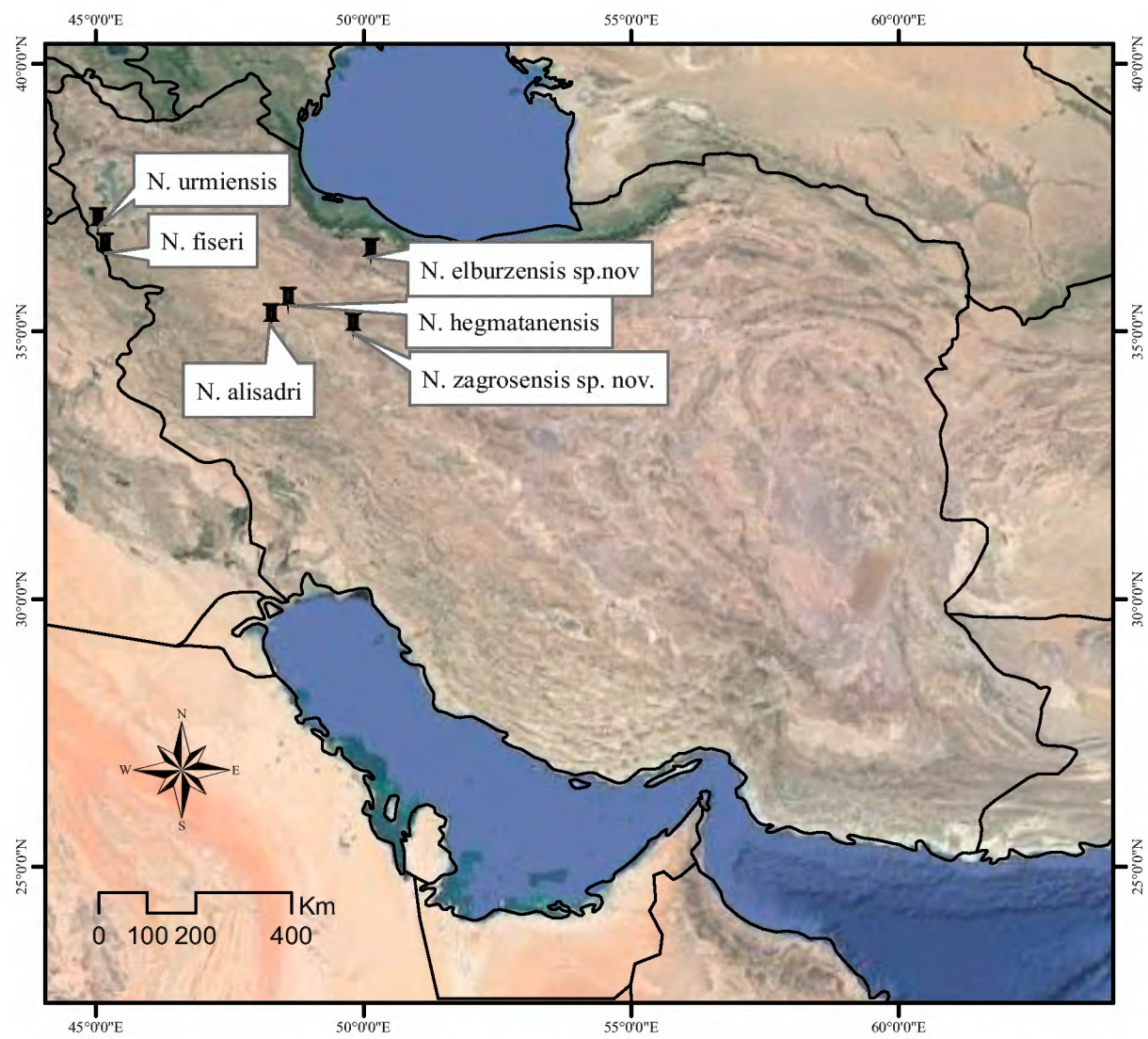

Figure 1. Map showing the type locality of *N. elburzensis* sp. nov. and *N. zagrosensis* sp. nov. along the Elburz and Zagros Mountains of Iran.

of 94 °C for 45 seconds, 55 °C for 30 seconds, 72 °C for 1 minute, and a final extension of 72 °C for 7 minutes. PCR products were purified and sequenced by Macrogen Inc. (Korea), using the primer pairs mentioned above.

The chromatograms were edited in BIOEDIT, version 7.0.5.3. The acquired sequences (with GenBank accession numbers PP496406 to PP496411 for COI and PP495485 to PP495490 for 28S) were analysed within the data set of Esmaeili-Rineh et al. (2015, 2017a) and Bargrizaneh et al. (2021), to identify the phylogenetic position of the newly collected specimens. The out-groups were selected based on previous phylogenetic analyses. The NCBI available sequences for *Niphargus krameri* Schellenberg, 1935, *Niphargus aquilex* Schiödte, 1855 and *Niphargus schellenbergi* S. Karaman, 1932 were used as outgroup (accession numbers: EF617274 and KF719253, EF617264 and JF420841, JF420854 and EU693321). All sequences were edited and aligned using CLUSTALW (Thompson et al. 1994), as implemented in the BIOEDIT, version 7.0.5.3, program sequence alignment editor (Hall 1999), using the default settings.

We conducted phylogenetic reconstruction using Bayesian inference in MRBAYES, version 3.1.2 (Ronquist and Huelsenbeck 2003). The Bayesian analyses were run for 15 million generations, employing the TIM3+G and TIM3+I+G models (selected using JMODELTEST, version 0.1.1, Posada 2008) for the 28S and COI genes, respectively. We ran four chains, with trees sampled every 1000 generations. The initial 3750 sampled trees were discarded as burn-in. We assessed tree likelihood convergence using Tracer 1.5.0 (Drummond and Rambaut 2009). A consensus tree representing the majority rule at fifty percent was computed using the remaining trees and visualized using FIGTREE v1.4.0 software. For detailed information about the analyzed species, please refer to the Electronic Supplement provided in Esmaeili-Rineh et al. (2015) and Bargrizaneh et al. (2021). To assess the genetic divergence from previously described Iranian species of *Niphargus*, we calculated genetic distances using the Kimura two-parameter (K2P) model (Kimura 1980), implemented in MEGA ver. 5 (Tamura et al. 2011).

Results

Phylogenetic position of the new species and their genetic distinctness

The results of genetic distance analysis strongly supported the species status of the *Niphargus* specimens collected in this study. Six individuals, three from Alamout and three from Kahak springs were sequenced and analyzed. The specimens from the Kahak population shared a single haplotype for a 902 base-pair segment of the 28S ribosomal DNA gene but had two haplotypes for a 513 base-pair segment of the COI gene. The specimens from the Alamout population displayed a unique haplotype for both of these genes. Herein studied specimens were nested within the Middle East clade as illustrated in Fig. 2.

For the 28S gene fragment, the pairwise K2P genetic distance among *N. zagrosensis* sp. nov. from Kahak spring and the other species ranged from a minimum of 0.13% (*N. alisadri*) to a maximum of 11.36% (*N. daniali*). The pairwise K2P genetic distance among *N. zagrosensis* sp. nov. and the other species ranged from a minimum of 5.73% (*N. alisadri*) to a maximum of 20.66% (*N. daniali*), based on COI gene fragment.

The pairwise K2P genetic distance among *N. elburzensis* sp. nov. and the other species ranged from a minimum of 1.56% (*N. urmiensis*) to a maximum of 10.98% (*N. daniali*), for the 28S gene fragment. The pairwise K2P genetic distance among *N. elburzensis* sp. nov. from Alamout spring and the other species ranged from a minimum of 10.70% (*N. fiseri*) to a maximum of 23.48% (*N. daniali*), based on COI gene fragment. The pairwise K2P genetic distance between two new species is 2.21% and 17.63% based on 28S and COI gene fragments, respectively. This indicates that the new species are well differentiated genetically. A comprehensive overview of the pairwise Kimura two-parameter genetic distances among the Iranian taxa is presented in Table 1.

Taxonomic part

Order Amphipoda Latreille, 1816
Family Niphargidae Bousfield, 1977
Genus *Niphargus* Schiödte, 1849

***Niphargus elburzensis* sp. nov.**
https://zoobank.org/DDB40AED-D8D2-42B2-A957-09F517DF2E30
Figs 3–6

Diagnosis (based on male only). Each telson lobe with five distal spines, two lateral spines and one mesial spine. The propodi of gnathopod II rectangular shape with two L-setae on palmar corner. Ventro-posterior corner of epimeral plates I to III produced. Outer plate of maxilla I with seven long spines with 2-2-1-1-3-0-1 denticles.

Etymology. The name "elburzensis" refers to Elburz Mountains in the north of Iran, where the species was found.

Material examined. ***Holotype.*** Iran • male; Qazvin Province, Northeastern Qazvin City, Alamout Spring; coordinates: 36°28.56'N, 50°8.52'E. Specimens collected by S.A. Mirghaffari; 25 July 2022. Holotype with two paratypes are stored under catalogue number ZCRU Amph. 1503.

Description of holotype. Total length of specimen 9 mm. Body strong. Head length 9% of body length. Lateral cephalic lobes sub-rounded (Fig. 3A).

Antennae I–II. Antenna I is 0.4 times body length. Peduncular articles 1–3 progressively shorter; length of peduncular article 3 exceeds half of peduncular article 2 (ratio 1.00: 1.80). Main flagellum with 20 articles (most with short setae). Accessory flagellum bi-articulated and reaching half of article 4 of main flagellum; articles with one and two setae, respectively (Fig. 3B).

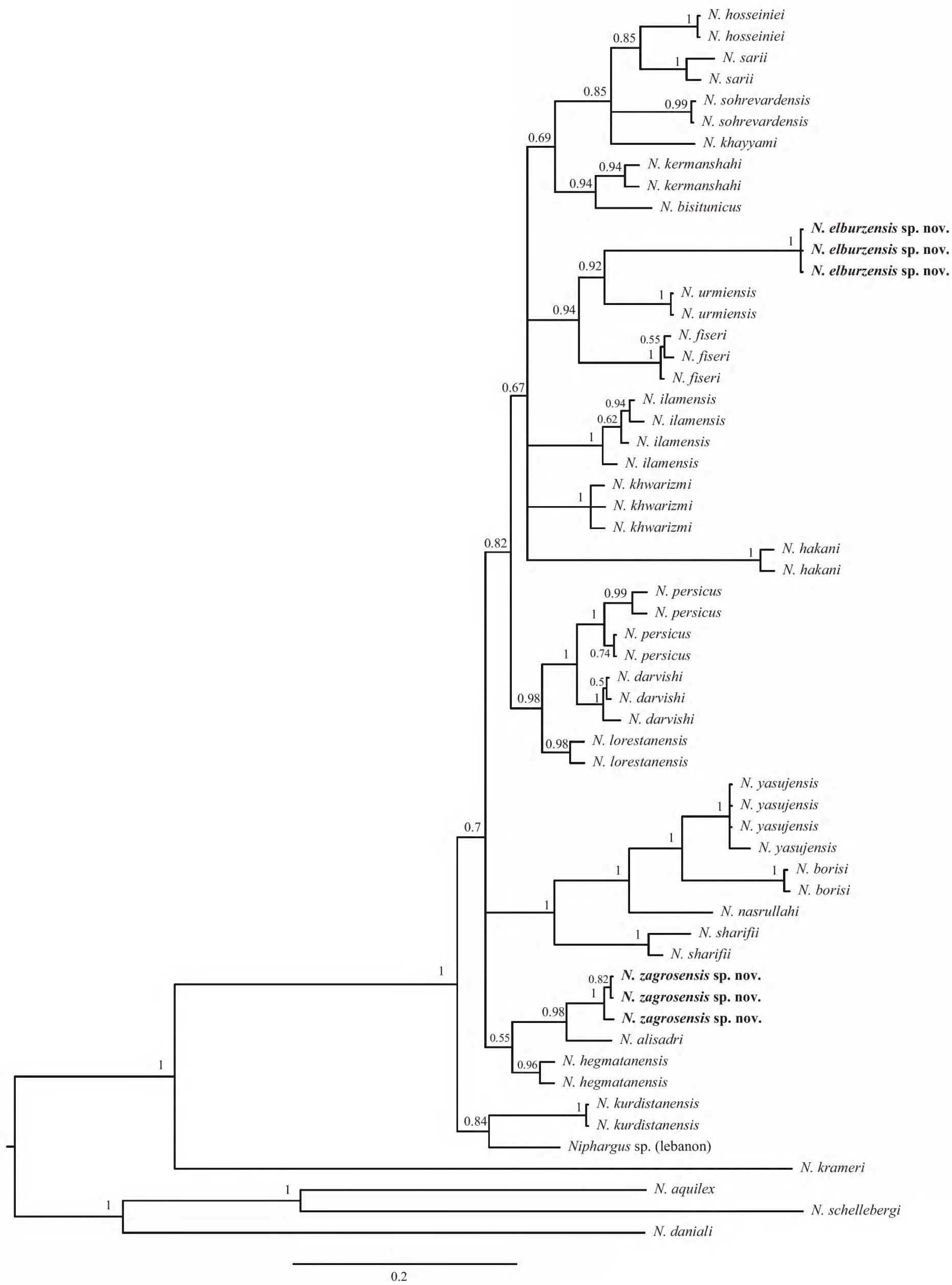

Figure 2. Bayesian consensus tree of 58 *Niphargus* Schiödte, 1849 specimens based on the 28S ribosomal DNA and COI gene sequences. Species are identified and named according to available taxonomic descriptions. Posterior probabilities are indicated on branches.

Table 1. Kimura 2-Parameter distances (K2P) comparing Iranian species with newly collected populations of the genus *Niphargus* Schiödte, 1849, based on 28S ribosomal DNA gene (below Diagonal) and mtDNA (COI) gene (above Diagonal).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1: *N. zagrosensis* sp.nov.		0.98	17.63	5.94	9.65	17.35	20.39	11.65	11.97	11.40	11.90	12.15	11.39	11.19	14.66	11.45	14.74	12.18	11.24	14.67	N/A	N/A	N/A	N/A
2: *N. zagrosensis* sp.nov.	0.00		17.89	5.73	10.34	17.60	20.66	11.89	12.45	12.34	12.61	12.87	11.62	11.66	15.15	11.69	14.99	12.66	11.72	14.92	N/A	N/A	N/A	N/A
3: *N. elburzensis* sp.nov.	2.21	2.21		15.60	13.72	16.06	23.48	13.21	10.70	16.81	14.61	13.91	15.80	14.61	17.28	13.44	15.59	18.67	10.91	17.59	N/A	N/A	N/A	N/A
4: *N. alisadri*	0.13	0.13	2.35		11.03	16.88	20.91	12.35	12.16	11.9	13.08	12.38	12.1	12.36	15.41	12.36	15.76	14.15	12.15	15.93	N/A	N/A	N/A	N/A
5: *N. bisitunicus*	1.03	1.03	2.21	1.15		14.7	21.67	10.49	10.72	10.51	11.16	11.16	12.07	12.8	14.9	8.7	14.71	13.6	9.8	13.5	N/A	N/A	N/A	N/A
6: *N. borisi*	2.33	2.33	3.41	2.17	2.7		23.23	13.19	14.12	17.07	15.34	15.82	14.88	14.37	10.04	13.9	12.76	17.86	13.91	7.38	N/A	N/A	N/A	N/A
7: *N. daniali*	11.36	11.36	10.98	11.2	11.67	11.49		17.12	19.62	22.18	21.96	19.89	19.87	17.06	23.05	17.82	22.18	21.7	18.59	21.93	N/A	N/A	N/A	N/A
8: *N. darvishi*	0.90	0.90	2.61	0.76	1.4	2.69	11.51		9.58	12.08	10.27	9.82	12.27	10.01	13.65	3.82	14.86	13.33	8.69	11.8	N/A	N/A	N/A	N/A
9: *N. fiseri*	1.42	1.42	1.96	2.57	2.96	4.29	11.47	3.09		12.52	12.98	11.37	11.59	11.37	12.98	10.02	13.95	13.78	7.62	15.14	N/A	N/A	N/A	N/A
10: *N. hosseiniei*	1.29	1.29	2.08	1.4	1.27	2.82	12.28	1.4	2.83		13.99	12.1	13.22	14.18	13.44	12.09	15.65	9.65	13.7	14.16	N/A	N/A	N/A	N/A
11: *N. ilamensis*	1.16	1.16	2.35	1.27	1.15	2.83	11.84	1.27	2.83	0.89		8.98	12.52	11.87	15.61	10.07	14.67	13.31	10.02	14.42	N/A	N/A	N/A	N/A
12: *N. khwarizmi*	0.90	0.90	2.21	1.02	1.15	2.56	11.99	1.27	2.83	1.14	1.02		11.61	10.52	13.43	8.95	15.85	15.53	9.13	16.09	N/A	N/A	N/A	N/A
13: *N. kurdistanensis*	0.77	0.77	2.48	0.63	1.27	2.04	11.05	1.14	2.96	1.66	1.66	1.4		9.78	13.7	11.58	14.7	15.44	12.05	14.42	N/A	N/A	N/A	N/A
14: *Niphargus* sp. (Lebanon)	0.77	0.77	2.48	0.63	1.53	2.04	11.37	1.14	2.97	1.66	1.66	1.4	0.25		15.82	9.57	15.61	16.16	9.82	14.62	N/A	N/A	N/A	N/A
15: *N. nasrullahi*	1.03	1.03	2.75	0.89	1.53	1.78	11.36	1.4	3.23	1.79	1.66	1.4	1.27	1.28		13.9	14.99	16.94	13.22	8.98	N/A	N/A	N/A	N/A
16: *N. persicus*	1.03	1.03	2.75	0.89	1.53	2.82	11.67	0.25	3.23	1.53	1.4	1.4	1.27	1.27	1.53		14.87	14.33	8.9	12.98	N/A	N/A	N/A	N/A
17: *N. sharifii*	0.77	0.77	2.48	0.63	1.02	1.78	11.05	1.14	2.96	1.53	1.4	1.15	0.76	0.76	0.76	1.27		15.71	13.7	14.48	N/A	N/A	N/A	N/A
18: *N. sohrevardensis*	0.90	0.90	2.08	1.02	0.89	2.56	12.1	1.01	2.56	0.38	0.51	0.76	1.4	1.4	1.4	1.14	1.15		14.72	16.42	N/A	N/A	N/A	N/A
19: *N. urmiensis*	2.20	2.20	1.56	2.33	2.2	3.79	11.61	2.59	2.06	2.59	2.6	2.46	2.46	2.47	2.73	2.73	2.2	2.46		13.47	N/A	N/A	N/A	N/A
20: *N. yasujensis*	1.81	1.81	3.29	1.66	2.31	1.14	11.21	2.18	4.03	2.57	2.44	2.18	1.79	1.79	1.27	2.31	1.27	2.18	3.26		N/A	N/A	N/A	N/A
21: *N. hakani*	1.68	1.68	2.22	2.43	2.56	3.88	11.76	2.95	2.83	2.56	2.43	2.3	2.83	2.83	2.83	3.09	2.56	2.17	2.2	3.35		N/A	N/A	N/A
22: *N. hegmatanensis*	0.38	0.38	2.21	0.25	1.15	1.91	11.51	0.76	2.83	1.4	1.27	1.02	0.63	0.63	0.89	0.89	0.63	1.02	2.33	1.4	2.43		N/A	N/A
23: *N. kermanshahi*	0.77	0.77	2.21	0.89	0.51	2.43	11.67	1.14	2.96	1.01	0.89	0.63	1.02	1.28	1.27	1.27	1.02	0.63	2.46	2.05	2.3	0.89	N/A	N/A
24: *N. lorestanensis*	0.51	0.51	2.48	0.38	1.27	2.56	11.36	0.38	2.7	1.27	1.15	1.15	1.02	1.02	1.27	0.5	1.02	0.89	2.46	2.05	2.83	0.63	1.02	
25: *N. sarii*	1.29	1.29	2.21	1.4	1.27	2.96	12.28	1.4	3.23	0.76	1.14	1.14	1.79	1.79	1.79	1.53	1.53	0.63	2.73	2.57	2.82	1.4	1.01	1.27

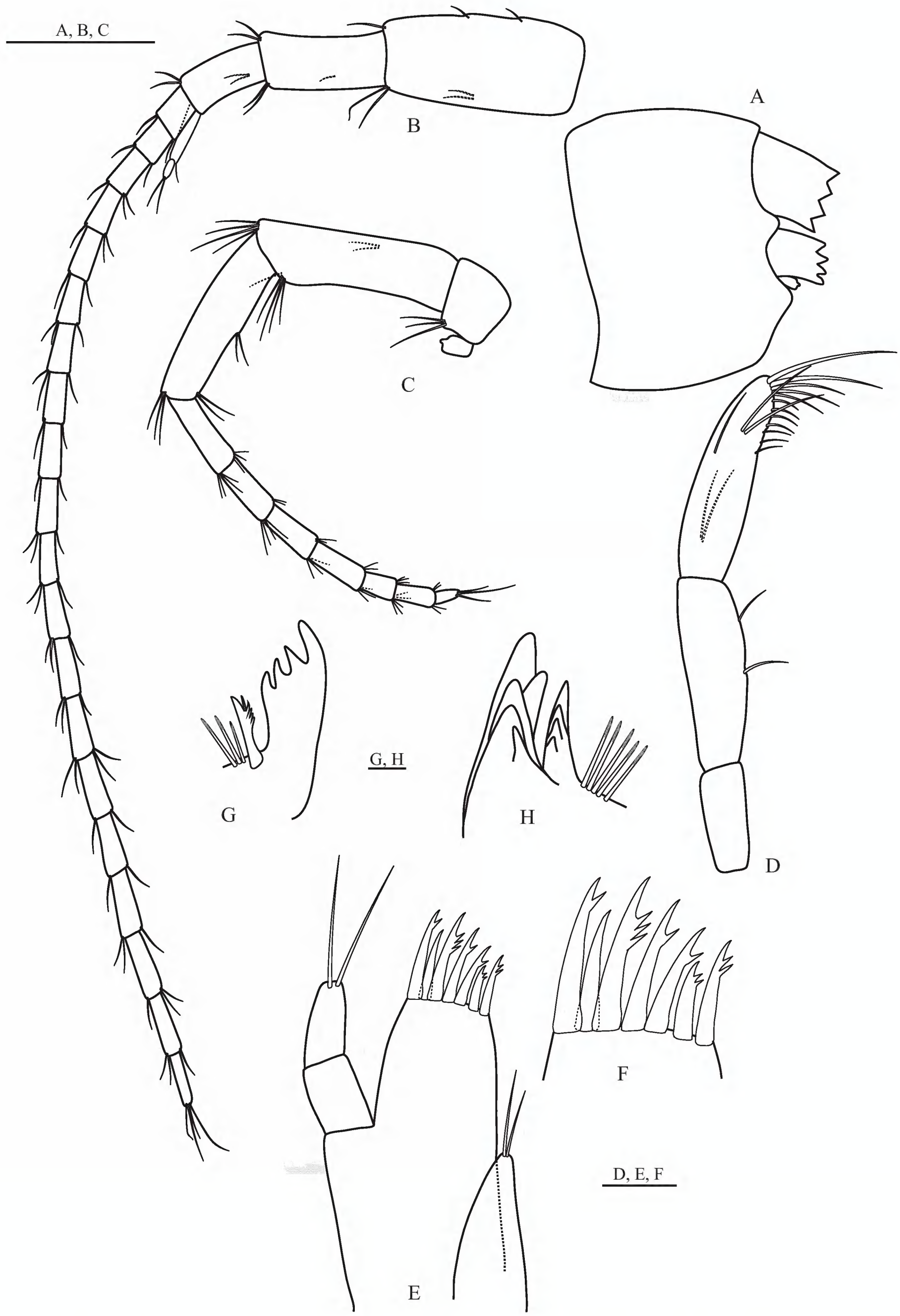

Figure 3. *Niphargus elburzensis* sp. nov., holotype, male, 9 mm (ZCRU Amph.1503). **A.** Head; **B.** Antenna I; **C.** Antenna II; **D.** Mandibular palp; **E, F.** Maxilla I; **G.** Right mandible; **H.** Left mandible. Scale bars: 0.25 mm (**G–H**); 0.5 mm (**A, D–F**); 1 mm (**B, C**).

Antenna II with flagellum formed of seven articles. Length ratio antenna I: II as 1: 0.49. Flagellum length is 0.9 times length of peduncle articles 4 + 5. Peduncular article 4 of antenna II is longer than article 5 (1.3: 1.00), peduncle articles 4 and 5, with three groups of simple setae each (Fig. 3C).

Mouthparts. Labium bi-lobate; both lobes with numerous fine distal and lateral setae (Fig. 4D). Mandible: right mandible with four teeth on incisor process, lacinia mobilis pluritoothed, between pars incisiva and pars molaris a row of three setae with lateral projections (Fig. 3G). Left mandible with five teeth on incisor process, lacinia mobilis with four teeth, between pars incisiva and pars molaris a row of five setae with lateral projections (Fig. 3H). Mandibular palp articles 1:2:3 represent 20%, 37% and 43% of total palp length, respectively. Proximal article without setae; second article with two setae along ventral margin and third article with one group of two A-setae, two groups of B-setae, no C-setae, eight D-setae and four E-setae (Fig. 3D). Inner plate of maxilla I with two long apical setae, outer plate with seven long spines with 2-2-1-1-3-0-1 denticles; palp bi-articulated, slightly longer than outer lobe, with two apical setae (Fig. 3 E, F). Both plates of maxilla II with numerous long distal setae (Fig. 4C). Maxilliped with short inner plate bearing three distal spines intermixed with four distal setae and two lateral setae sub-distally; outer plate exceeding half of palp article 2, with nine spines along inner margin and three setae distally; Maxilliped palp article 3 with one group of simple setae at outer and inner margins; palp terminal article with one simple seta at outer margin, nail shorter than pedestal (Fig. 4E).

Gnathopods. Coxal plate of gnathopod I shorter than gnathopod II. Coxa of gnathopod I rectangular, broader than long, anterior and ventral margins with five setae. Basis with several single setae on anterior and posterior margins; ischium and merus with one posterior group of setae. Carpus with one group of five setae anterio-distally, bulge with setae; carpus 0.58 times basis length and 0.89 times propodus length. Propodus of gnathopod I trapezoid shape and broader than long; anterior margin with five setae in one group in addition to antero-distal group of four setae. Palm slightly convex, defined by one strong long corner S-seta accompanied laterally by three L-setae with lateral projections, on inner surface of palmar corner one short sub-corner R-seta. Dactylus reaching the posterior margin of propodus, outer and inner margins of dactylus with one and two simple setae, respectively. Nail length 0.33 times total dactylus length (Fig. 4A). Coxal plate of gnathopod II of rectangular shape, longer than broad, anterio-ventral margin with five setae. Basis with single setae on anterior and posterior margins; ischium and merus with one posterior group of setae. Carpus with one group of three setae antero-distally, bulge with long setae; carpus 0.7 times basis length and 0.90 times propodus length. Propodus of gnathopod II of rectangular shape, longer than broad; anterior margin with three setae in two groups in addition to antero-distal group of four setae. Palm slightly convex, defined by one strong long corner S-seta accompanied laterally by two L-setae with lateral projections, on inner surface of palmar corner one short sub-corner R-seta. Dactylus reaching the posterior margin of propodus, outer and inner margins of dactylus with one and two setae, respectively; nail short, 0.3 times total dactylus length (Fig. 4B).

Pereonites I–VII. Without setae.

Pereopods. Coxal plate III rectangular, length to width ratio is 1.07: 1; antero-ventral margin with four simple setae. Coxal plate IV rectangular, antero-ventral margin with five setae, posterior concavity shallow and approximately 0.1 times coxa width. Coxal plate V–VI with large anterior lobe; Coxal plate V with three setae on anterior and posterior lobes each. Coxal plate VI with two setae on anterior lobe. Coxal plate VII with one seta on posterior margin (Fig. 5A–E).

***Pereopod III*:** IV length ratio is 1: 1.03. Dactylus III length 0.39 times propodus length, nail shorter than pedestal. Dactylus IV length 0.32 times propodus length, nail shorter than pedestal (Fig. 5A, B). Pereopods length V: VI: VII ratios as 1: 1.2: 1.36, respectively (Fig. 5C–E). Pereopod VII 0.48 times total body length. Pereopod bases V–VII with five, six and five groups of spines along anterior margins and with six, 10 and nine setae along posterior margins, respectively. Antero-ventral lobe of ischium in pereopods V–VII slightly developed. Merus and carpus in pereopods V–VII with several groups of spines and setae along anterior and posterior margins; propodus of pereopod VII longer than these in V–VI, dactyli in pereopods V–VII with one seta on outer margin, nail length of pereopod VII 0.34 times total dactylus length (Fig. 5C–E).

Pleonites I–III. Each with one seta along the dorsal margin.

Pleopods. Peduncle of pleopods I–III with two-hooked retinacles distally; Peduncle of pleopod III with two setae along of inner margin. Rami of pleopods I–III with five to eight articles (Fig. 6A–C).

Urosomites I–III. At the base of uropod I with one strong spine; Urosomites I-II postero-dorso-laterally with one and two spines, respectively. Urosomite II with two simple setae on postero-dorso-laterally. Urosomite III without setae.

Uropods. Peduncle of uropod I with six and five large spines along dorso–lateral and dorso–medial margins, respectively. Inner ramus of uropod I longer than outer ramus (ratio 1: 1.05); inner ramus with five groups of spines laterally and five spines distally; outer ramus with three groups of five spines laterally and five spines distally (Fig. 6D). Inner ramus in uropod II longer than outer, both rami with lateral and distal long spines (Fig. 6E). Uropod III normal, almost 0.2 of body length. Peduncle of uropod III with five spines on distal margin. Outer ramus bi-articulated, distal article 0.11 times proximal article. The proximal article of outer ramus bearing five and four groups of spines along inner and outer margins, respectively; distal article with setae laterally and four setae distally. Inner ramus normal, with three distal spines (Fig. 6F).

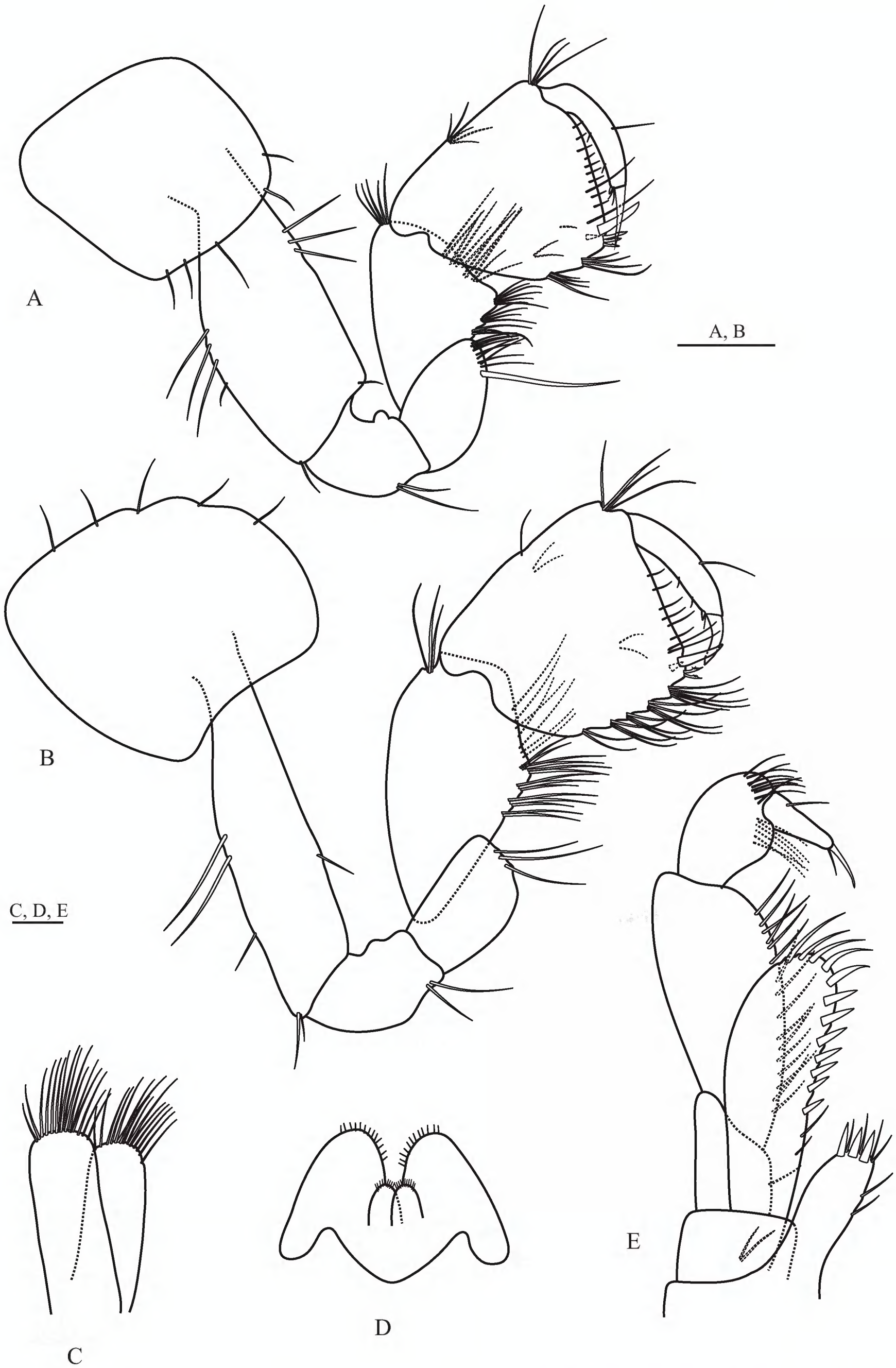

Figure 4. *Niphargus elburzensis* sp. nov., holotype, male, 9 mm (ZCRU Amph.1503). **A.** Gnathopod I; **B.** Gnathopod II; **C.** Maxilla II; **D.** Labium; **E.** Maxilliped. Scale bars: 0.5 mm (**C–E**); 1 mm (**A, B**).

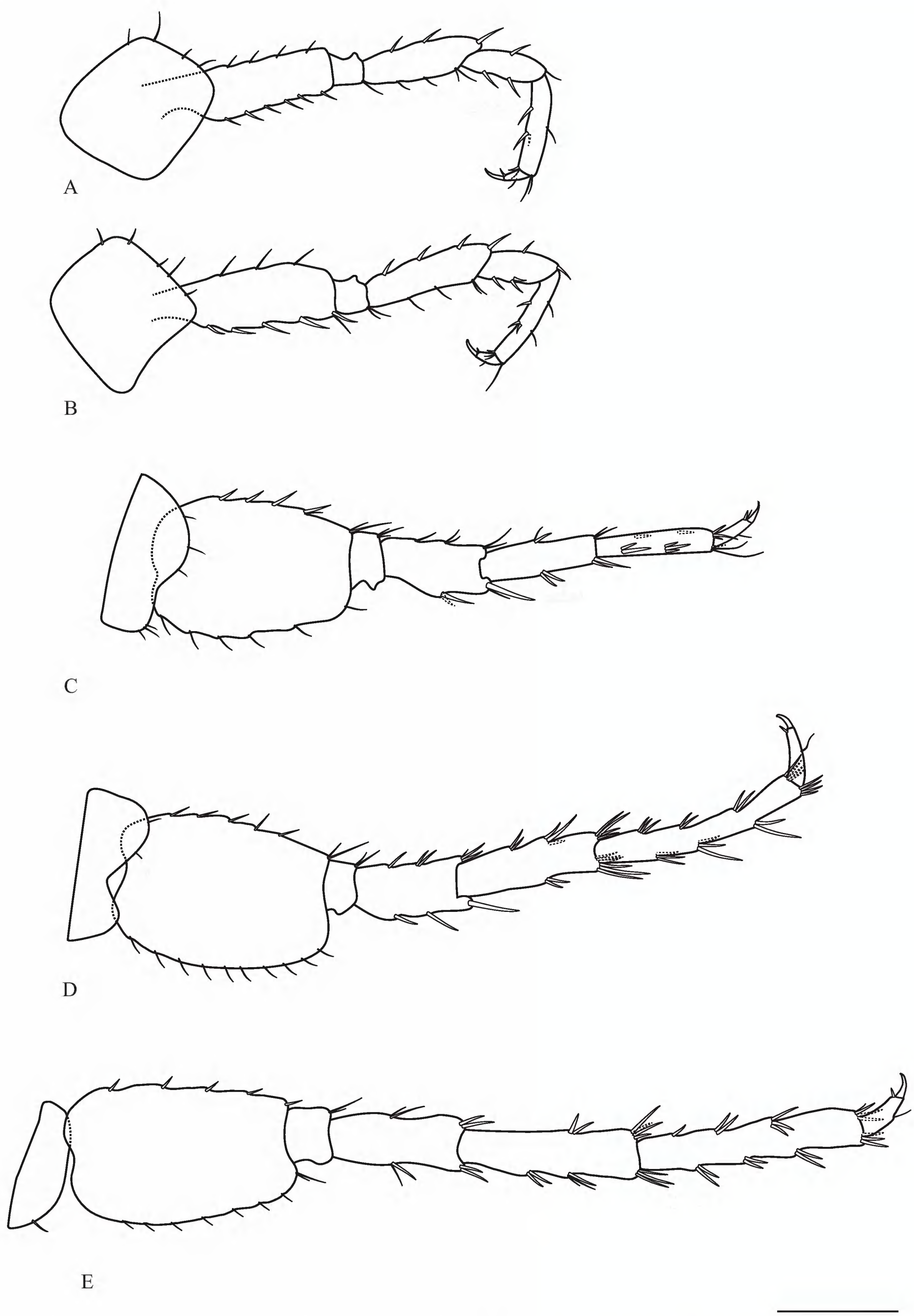

Figure 5. *Niphargus elburzensis* sp. nov., holotype, male, 9 mm (ZCRU Amph.1503). **A.** Pereopod III; **B.** Pereopod IV; **C.** Pereopod V; **D.** Pereopod VI; **E.** Pereopod VII. Scale bar: 1 mm.

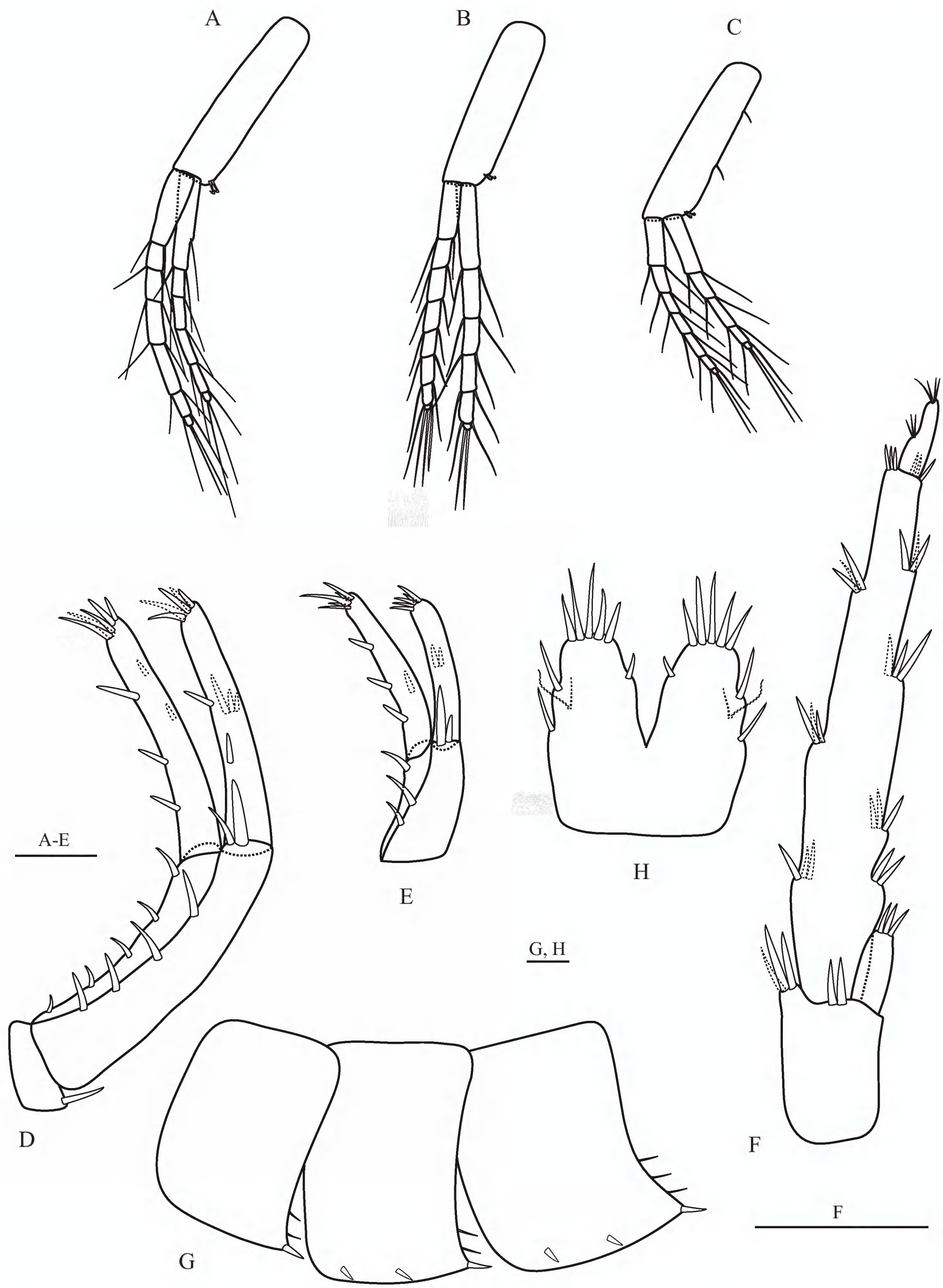

Figure 6. *Niphargus elburzensis* sp. nov., holotype, male, 9 mm (ZCRU Amph.1503). **A.** Pleopod I; **B.** Pleopod II; **C.** Pleopod III; **D.** Uropod I; **E.** Uropod II; **F.** Uropod III; **G.** Epimeral plates I–III; H. Telson. Scale bars: 0.5 mm (**G, H**); 1 mm (**A–E**); 2 mm (**F**).

Epimeral plates I–III. With angular postero-ventral corners, plates I–III posteriorly with three, three and four setae and spines, respectively. Epimeral plates II–III each with two spines along of ventral margin (Fig. 6G).

Telson. Telson length as long as broad; each lobe with five spines distally, with two long spines and two setae laterally, with one spine mesially (Fig. 6H).

Female. Unknown.

Genus *Niphargus* Schiödte, 1849

Niphargus zagrosensis sp.nov.

https://zoobank.org/27331B3E-2F2A-4DE4-ACA9-7EFA51D7B453

Figs 7–10

Diagnosis (based on male only). The propodi of gnathopod II triangular shape. Gnathopod II dactylus does not reach posterior margin of palm. Pereopod VI longer than pereopod VII. Maxilliped outer plate does not reach half of the posterior margin of palp article 2. Uropod III distal article exceeds more than 80% times proximal article. Outer plate of maxilla I with seven long spines with 4-2-1-2-1-1-0 denticles.

Etymology. The name "zagrosensis" refers to Zagros Mountains in the west of Iran, where the species was found.

Material examined. ***Holotype.*** Iran • male; Markazi Province, 54 km to Saveh City, Kahak Spring; coordinates 35°5.28'N, 49°49.02'E. Specimens collected by S.A. Mirghaffari; 20 July 2022. Holotype with two paratypes are stored under catalogue number ZCRU Amph. 1501.

Description of holotype. The total length of specimen 10.5 mm. Body strong and stout. Head length 10.5% of body length. Lateral cephalic lobes sub-rounded (Fig. 7A).

Antennae I–II. Antennae I 0.45 times body length. Peduncular articles 1–3 progressively shorter; length of peduncular article 3 not exceeds half of peduncular article 2 (ratio 1: 0.4). Main flagellum with 23 articles (most of which with short setae); accessory flagellum biarticulated and reaching 1/3 of article 4 of main flagellum, articles with two simple setae each (Fig. 7B). Antennae II flagellum with 11 articles, approximately 0.54 as long as antenna I. Peduncular article 4 longer than article 5, with 11 and 10 groups of simple setae, respectively. Flagellum length is 0.71 peduncle article 4 + 5 (Fig. 7C).

Mouthparts. Labium bi-lobate; with fine setae on tip of both lobes (Fig. 8D). Right mandible with four teeth on incisor process, lacinia mobilis pluritoothed; between pars incisiva and pars molaris a row of six setae with lateral projections (Fig. 7G). Left mandible with five teeth, lacinia mobilis with four teeth; between pars incisiva and pars molaris a row of six setae with lateral projections (Fig. 7H). Mandibular palp articles 1:2:3 represent 19%, 40% and 41% of total palp length, respectively. The proximal article has no setae, the second article with seven setae along ventral margin and the third article with one group of two A-setae, three groups of B-setae, no C-setae, 14 D-setae and five E-setae (Fig. 7D). Inner plate of maxilla I with two long distal simple setae; outer plate with seven long spines with 4-2-1-2-1-1-0 denticles; palp bi-articulated, slightly longer than the tip of outer lobe, with three long distal simple setae (Fig. 7E, F). Maxilla II bi-lobate (Fig. 8C). Both plates of maxilla II with numerous distal simple setae. Inner lobe with lateral simple setae (Fig. 7C). Maxilliped with short inner plate bearing four distal spines intermixed with six distal simple setae; outer plate less than half of the posterior margin of palp article 2, with 10 spines along inner margin and seven setae distally. Maxilliped palp article 3 with one proximal and one distal group of long simple setae at outer margin; palp terminal article with one seta at outer margin and two small setae at base of nail, nail shorter than pedestal (Fig. 8E).

Gnathopods. Coxa of gnathopod I shorter than gnathopod II. Coxal plate of gnathopod I trapezoid shape, ventro-posterior margins with 12 simple setae. Basis with several setae on anterior and posterior margins; ischium and merus with one posterior group of setae each. Carpus with one group of four setae antero-distally, a bulge with long simple setae; carpus 0.7 times basis length and 0.75 times propodus length. Propodus of gnathopod I rectangular shape; anterior margin with one group of five setae in addition to antero-distal group of five simple setae. Palm slightly convex, defined by one strong long corner S-seta accompanied laterally by three L-setae with lateral projections, on inner surface of palmar corner one short sub-corner R-seta. Dactylus reaching the posterior margin of propodus, outer and inner margins of dactylus with a row of two and three simple setae, respectively; nail short, 0.33 of total dactylus length (Fig. 8A).

Coxal plate of gnathopod II rectangular shape, with 13 setae along antero-ventro-posterior margins. Basis with single setae along anterior margin and with setae in group along posterior margin; ischium and merus with one posterior group of setae each. Carpus 0.4 times basis length and 0.59 times propodus length. Carpus with one group of four setae antero-distally. Propodus of gnathopod II larger than gnathopod I, triangle shape and broader than long; anterior margin with three setae in one group in addition to antero-distal group of three simple setae. Palm slightly convex, defined by one strong long corner S-seta accompanied laterally by two L-setae with lateral projections, on inner surface of palmar corner one short sub-corner R-seta. Dactylus does not reach the posterior margin of propodus, outer and inner margins of dactylus with two and three simple setae, respectively. Nail length 0.29 times total dactylus length (Fig. 8B).

Pereonites I–VII. Pereonites II and IV with one seta each; others pereonites without setae.

Pereopods. Coxal plate III rectangular shape, length to width ratio as 1.2: 1; antero-ventral margin with nine simple setae. Coxal plate IV rectangular shape, length to width ratio as 1: 1.02, antero-ventro-posterior margins with 12 simple setae, posterior concavity shallow and approximately 0.1 of coxa width. Coxal plate V with large anterior lobe, with five and three simple setae on anterior and posterior lobes, respectively. Coxal plate VI with anterior lobe, with four and three simple setae on anterior and posterior lobes, respectively. Coxal plate VII with one simple seta on posterior margin (Fig. 9A–E).

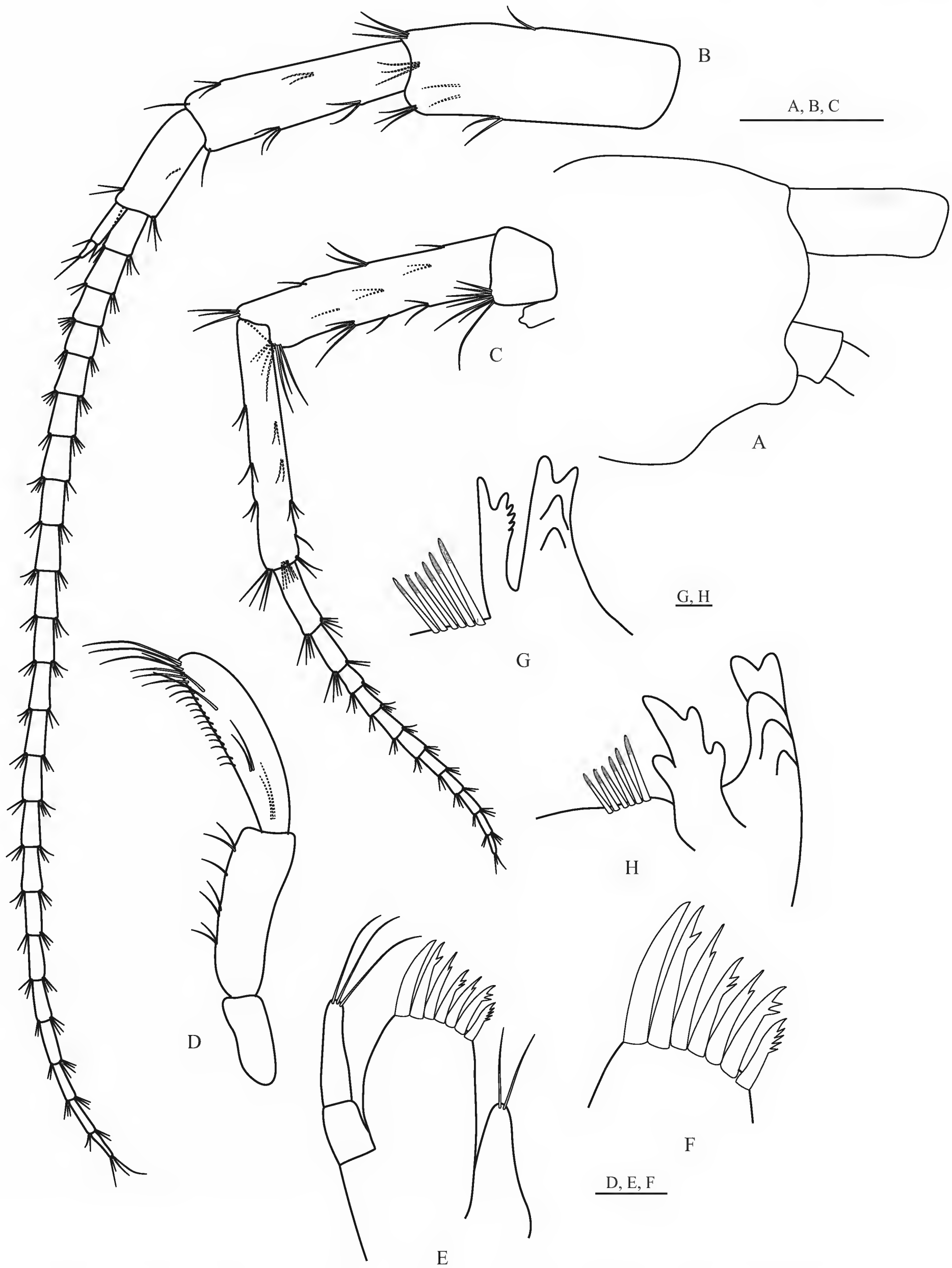

Figure 7. *Niphargus zagrosensis* sp. nov., holotype, male, 10.5 mm (ZCRU Amph.1501). **A.** Head; **B.** Antenna I; **C.** Antenna II; **D.** Mandibular palp; **E, F.** Maxilla I. **G.** Right mandible. **H.** Left mandible. Scale bars: 0.25 mm (**G–H**); 0.5 mm (**A, D–F**); 1 mm (**B, C**).

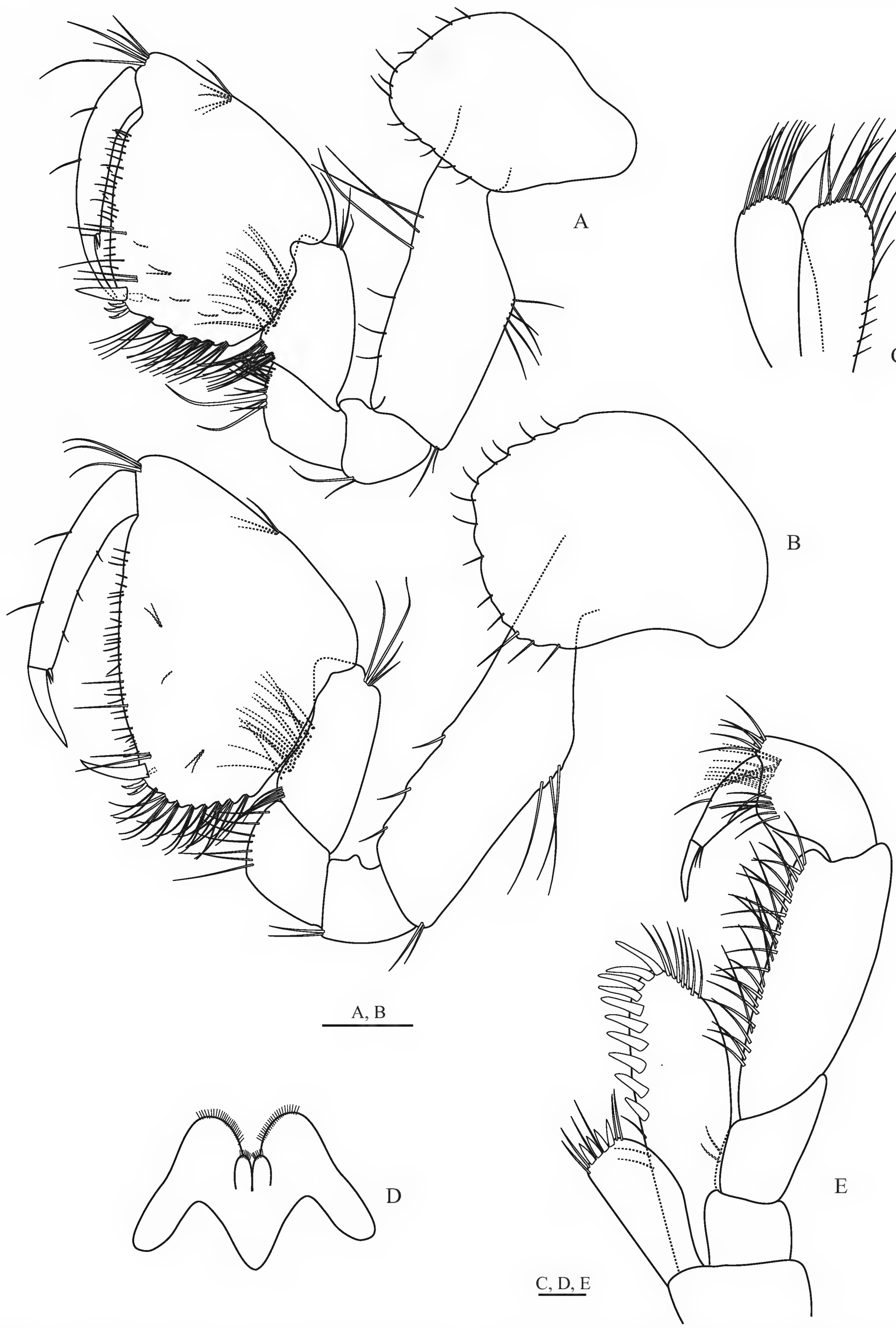

Figure 8. *Niphargus zagrosensis* sp. nov., holotype, male, 10.5 mm (ZCRU Amph.1501). **A.** Gnathopod I; **B.** Gnathopod II; **C.** Maxilla II; **D.** Labium; **E.** Maxilliped. Scale bars: 0.5 mm (**C–E**); 1 mm (**A, B**).

Pereopods length III: IV ratio is 1: 0.94. Dactylus III length 0.40 times propodus length, nail shorter than pedestal (Fig. 9A). Dactylus IV length 0.43 times propodus length, nail shorter than pedestal (Fig. 9B). Dactyli of pereopods III–IV with one seta on inner margin (Fig. 9A, B).

Pereopods length V: VI: VII ratios is 1: 1.28: 1.25, respectively. Pereopod VII 0.48 times total body length. Pereopod bases V–VII each with seven, seven and six groups of spines along anterior margins and 13, 13 and 14 simple setae along posterior margin, respectively. Ischium, merus and carpus in pereopods V–VII with several groups of spines and simple setae along anterior and posterior margins; propodus of pereopod VI longer than these in V and VII, dactyli of pereopods V–VII with one spine at the base of nail on inner margin, nail length of pereopod VII 0.33 times total dactylus length (Fig. 9A–E).

Epimeral plates I–III. With angular postero-ventral corner, anterior and ventral margins convex; postero-ventral corners of plates I-III with three, four and five spines and setae, respectively. Epimeral plates II–III with three and four spines along of ventral margin, respectively (Fig. 10G).

Pleonites I–III. With one simple seta along the dorsal margin each.

Pleopods I–III. Peduncle of pleopods I–III with two-hooked retinacles distally; peduncle of pleopod III with three simple setae along of inner margin. Rami of pleopods I–III with nine to 14 articles (Fig. 10A–C).

Urosomites I–III. At the base of uropod I with one strong spine. Urosomites I and II with one and three spines on postero-distally, respectively. Urosomite III without setae.

Uropods I–III. Peduncle of uropod I with seven and four large spines along dorso-lateral and dorso-medial margins, respectively. Inner ramus of uropod I longer than outer ramus (ratio 1: 1.06). Inner ramus with five groups of spines laterally and five spines distally; outer ramus with four groups of six spines laterally and five spines distally (Fig. 10D). Inner ramus in uropod II longer than outer, both rami with lateral and distal long spines (Fig. 10E). Uropod III long, almost 0.44 times body length. Peduncle of uropod III with four spines on distal margin. Outer ramus bi-articulated, distal article 87% times proximal article. The proximal article of outer ramus bearing five and four groups of spines along of outer and inner margins, respectively; distal article with several groups of simple setae laterally and four simple setae distally. Inner ramus short, with two distal spines (Fig. 10F).

Telson. Telson longer than broad; lobes slightly narrowing; each lobe with four spines apically, with two spines and two simple setae laterally (Fig. 10H).

Female. Unknown.

Intraspecific variation

Intraspecific variabilities of each species were investigated by three individuals. In *N. elburzensis*, only a difference in the number of L-setae in gnathopod I (ranging between 2–3) was found among individuals. In *N. zagrosensis* was observed a greater number of intraspecific differences. These differences included the number of apical spines in each telson lobe (between 3–4), the ratio of antennal length II to I, and the ratio of segments 4 + 5 to the flagellum length in antenna II. It's important to note that the diagnostic characteristics of each species are based on the fixed characters, which exhibit a consistent state for all individuals of a species.

Discussion

In this research, two populations of the genus *Niphargus* were collected from Iran and examined based on morphological and molecular characteristics. DNA sequences support the species status of two new species, *N. elburzensis* sp. nov. and *N. zagrosensis* sp. nov. The Bayesian analysis revealed that the two newly identified species are phylogenetically separate and independent lineages, as indicated by high bootstrap values.

Niphargus elburzensis sp. nov. is characterized by two clearly visible characters. The first one is the presence of five distal, two lateral and one mesial spines on each telson lobe. Although the presence of a mesial spine on the telson lobe is common among European species (for example *N. podogoricensis* Karaman, 1950; *N. vinodolensis* Fišer, Sket & Stoch, 2006), this trait was observed only in *N. arasbaranensis* (in press) and *N. elburzensis* between Iranian species. However, *N. elburzensis* is distinguished from *N. arasbaranensis* by the presence of five distal spines on each telson lobe (compared to four distal spines in *N. arasbaranensis*) and the greater length of the palpus to the tip of the outer lobe in maxilla I (compared to equal length of the palpus and outer plate in *N. arasbaranensis*). The second distinguishing characteristic involves the presence of two setae along the inner margin of pleopod III. We found this trait in three species in Iran. However, neither *N. kurdistanensis*, *N. urmiensis* nor *N. fiseri* share produced epimeral plates (Mamaghani-Shishvan et al. 2017; Mamaghani-Shishvan and Esmaeili-Rineh 2019).

Although *N. elburzensis* is genetically close to *N. fiseri* and *N. urmiensis*, it differs from these two species by several characteristics. These differences include a long palpus in maxilla I, which passes from the tip of the outer lobe, in contrast to *N. urmiensis* and *N. fiseri* exhibit equal lengths of the palpus and outer plate. Moreover, *N. elburzensis* exhibit two and three L-setae on palmar corner of both gnathopods, whereas *N. urmiensis* and *N. fiseri* each bear only one L-seta on palmar corner of their gnathopods. Additionally, *N. elburzensis* is distinguished by the presence of five distal spines on each telson lobe, while *N. urmiensis* and *N. fiseri* each have three distal spines on each telson lobe. *N. elburzensis* and its sister taxa *N. urmiensis* and *N. fiseri* inhabit the shallow subterranean habitats. Geographic distances between the new species and its sister taxa are 462–448 km, respectively.

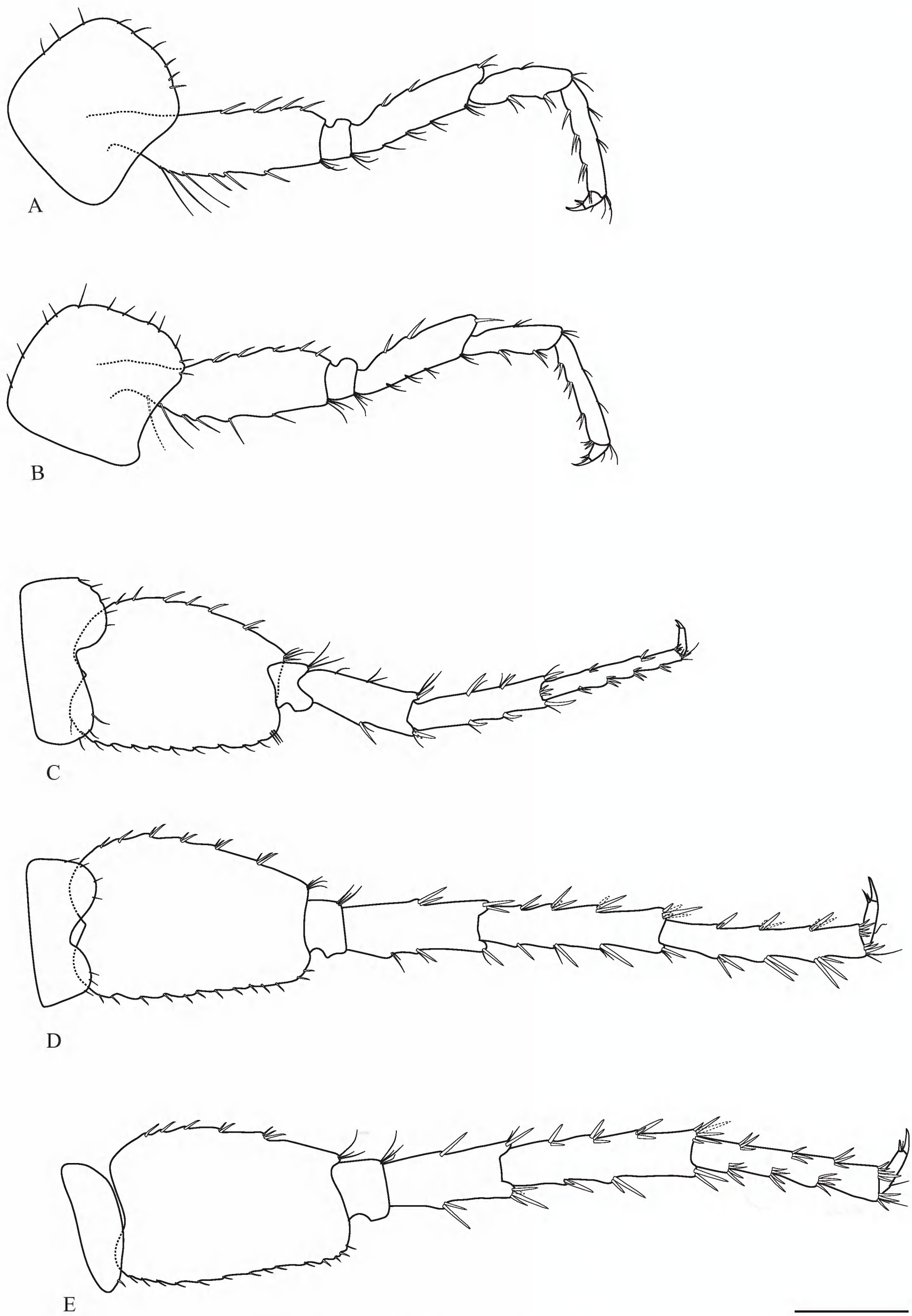

Figure 9. *Niphargus zagrosensis* sp. nov., holotype, male, 10.5 mm (ZCRU Amph.1501). **A.** Pereopod III; **B.** Pereopod IV; **C.** Pereopod V; **D.** Pereopod VI; **E.** Pereopod VII. Scale bar: 1 mm.

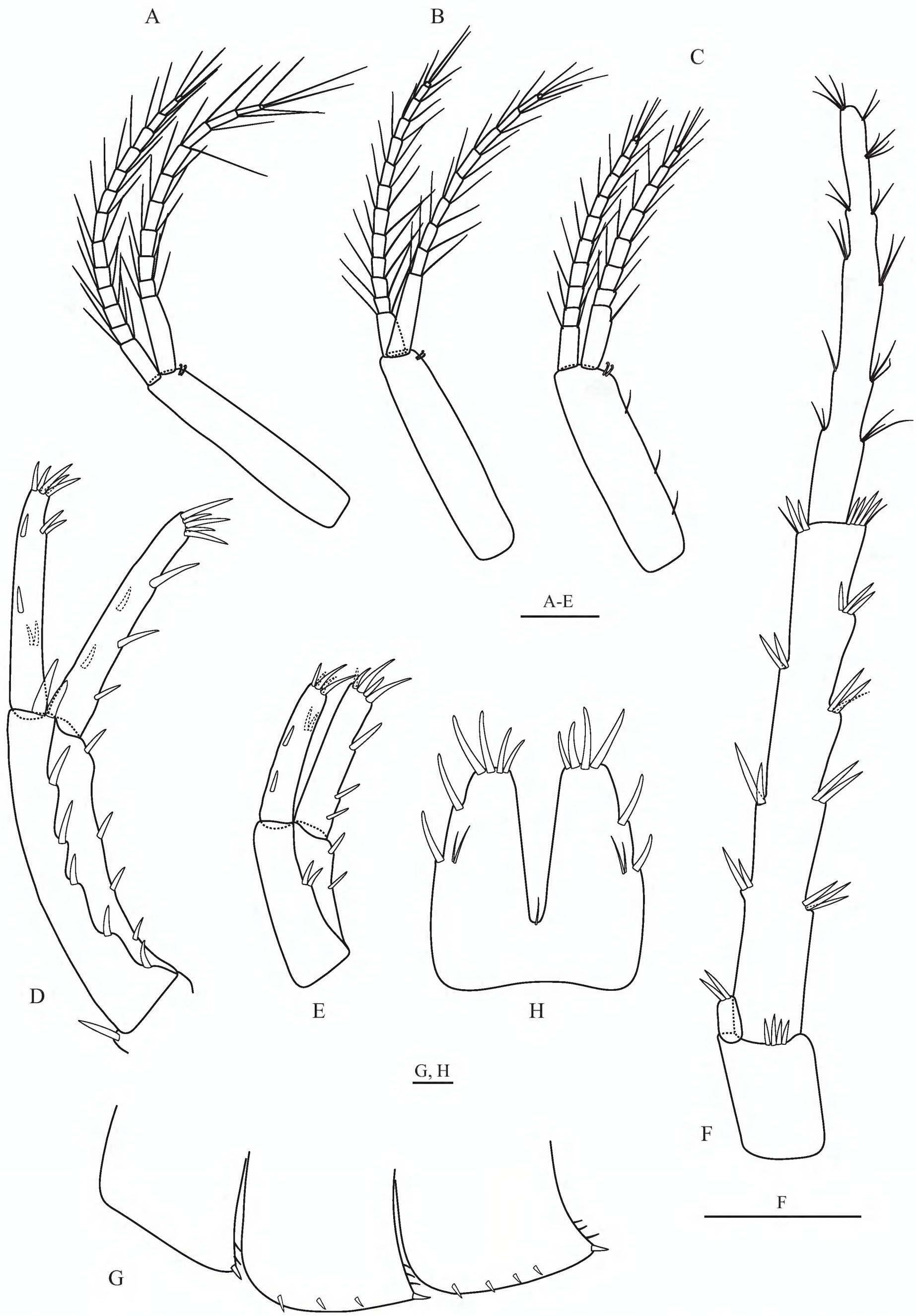

Figure 10. *Niphargus zagrosensis* sp. nov., holotype, male, 10.5 mm (ZCRU Amph.1501). **A.** Pleopod I; **B.** Pleopod II; **C.** Pleopod III; **D.** Uropod I; **E.** Uropod II; **F.** Uropod III; **G.** Epimeral plates I–III; **H.** Telson. Scale bars: 0.5 mm (**G, H**); 1 mm (**A–E**); 2 mm (**F**).

Niphargus zagrosensis is characterized by a distinctive combination of features, including a longer size of pereopod VI to pereopod VII and the maxilliped outer plate length less than half of posterior margin of palp article 2.

Although *N. zagrosensis* is genetically close to *N. alisadri*, it differs from *N. alisadri* in several morphological features: 1) Gnathopod II propodus shape: *N. zagrosensis* has a triangular shape, whereas *N. alisadri* has a rectangular shape. 2) Ratio of palpus to outer plate lobe length in maxilla I: *N. zagrosensis* has a long palpus in maxilla I, which passes from the tip of the outer lobe, while *N. alisadri* has a short palpus, which does not reach to maxilla I outer lobe. 3) L-setae in gnathopods I–II propodi: *N. zagrosensis* possesses three and two L-setae on gnathopods I–II propodi, respectively, while *N. alisadri* has two and zero L-setae (Esmaeili-Rineh and Sari 2013).

The sister taxa of *N. zagrosensis* are *N. alisadri* and *N. hegmatanensis*. While the new species and *N. hegmatanensis* inhabit shallow subterranean habitats, *N. alisadri* dwells in a cave lake habitat. The geographic distance between *N. zagrosensis* and *N. hegmatanensis* is 124 km, and between *N. zagrosensis* and *N. alisadri* is 141 km. Additionally, there is a geographic distance of 163 km between the two new species.

Recent studies suggest that the northern and western regions of Iran harbor considerable richness in groundwater amphipods (Mamaghani-Shishvan and Esmaeili-Rineh 2019; Bargrizaneh et al. 2021), highlighting the complexity of the ecosystem and its biodiversity. The presence of these organisms is crucial for nutrient cycling, energy flow, and overall ecosystem stability. Moreover, the abundance of amphipod species can serve as indicators of groundwater systems' health, providing valuable insights for environmental monitoring and conservation efforts.

Acknowledgments

This project was financially supported by Razi University as a part of the PhD thesis of the first author. We express our gratitude to editor Dr. Andrade and reviewers Dr. Cene Fišer and Dr. Denis Copilaş-Ciocianu for their valuable comments on the manuscript.

References

Astrin JJ, Stüben PE (2008) Phylogeny in cryptic weevils: molecules, morphology and new genera of western Palaearctic Cryptorhynchinae (Coleoptera: Curculionidae). Invertebrate Systematics 22(5): 503–522. https://doi.org/10.1071/IS07057

Balázs G, Borko Š, Angyal D, Zakšek V, Biró A, Fišer C, Herczeg G (2023) Not the Last Piece of the Puzzle: *Niphargus* Phylogeny in Hungary. Diversity 15(2): 223. https://doi.org/10.3390/d15020223

Bargrizaneh Z, Fišer C, Esmaeili-Rineh S (2021) Groundwater amphipods of the genus *Niphargus* Schiødte, 1834 in Boyer-Ahmad region (Iran) with description of two new species. Zoosystema 43(7): 127–144. https://doi.org/10.5252/zoosystema2021v43a7

Borko Š, Trontelj P, Seehausen O, Moškrič A, Fišer C (2021) A subterranean adaptive radiation of amphipods in Europe. Nature Communications 12(1): 1–12. https://doi.org/10.1038/s41467-021-24023-w

Copilaş-Ciocianu D, Boroş BV (2016) Contrasting life history strategies in a phylogenetically diverse community of freshwater amphipods (Crustacea: Malacostraca). Zoology : Analysis of Complex Systems, ZACS 119(1): 21–29. https://doi.org/10.1016/j.zool.2015.11.001

Drummond AJ, Rambaut A (2009) Bayesian evolutionary analysis by sampling trees. The phylogenetic handbook: a practical approach to phylogenetic analysis and hypothesis testing, 564–574. https://doi.org/10.1017/CBO9780511819049.020

Esmaeili-Rineh S, Sari A (2013) Two new species of *Niphargus* Schiödte, 1849 (Crustacea: Amphipoda: Niphargidae) from two caves in Iran. Journal of Natural History 47(41–42): 2649–2669. https://doi.org/10.1080/00222933.2013.802041

Esmaeili-Rineh S, Sari A, Delić T, Moškrič A, Fišer C (2015) Molecular phylogeny of the subterranean genus *Niphargus* (Crustacea: Amphipoda) in the Middle East: A comparison with European Niphargids. Zoological Journal of the Linnean Society 175(4): 812–826. https://doi.org/10.1111/zoj.12296

Esmaeili-Rineh S, Mirghaffari SA, Sharifi M (2017a) The description of a new species of *Niphargus* from Iran based on morphological and molecular data. Subterranean Biology 22: 43–58. https://doi.org/10.3897/subtbiol.22.11286

Fišer C, Trontelj P, Luštrik R, Sket B (2009) Toward a unified taxonomy of *Niphargus* (Crustacea: Amphipoda): a review of morphological variability. Zootaxa 2061(1): 1–22. https://doi.org/10.11646/zootaxa.2061.1.1

Fišer C, Alther R, Zaksek V, Borko S, Fuchs A, Altermatt F (2018) Translating *Niphargus* barcodes from Switzerland into taxonomy with a description of two new species (Amphipoda, Niphargidae). ZooKeys 760: 113–141. https://doi.org/10.3897/zookeys.760.24978

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In: Nucleic acids symposium series. Oxford, 95–98.

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16(2): 111–120. https://doi.org/10.1007/BF01731581

Mamaghani-Shishvan M, Esmaeili-Rineh S (2019) Two new species of groundwater amphipods of the genus *Niphargus* Schiödte, 1849 from northwestern Iran. European Journal of Taxonomy 546: 1–23. https://doi.org/10.5852/ejt.2019.546

Mamaghani-Shishvan M, Esmaeili-Rineh S, Fišer C (2017) An integrated morphological and molecular approach to a new species description of Amphipods in the Niphargidae from two caves in West of Iran. Zoological Studies (Taipei, Taiwan) 56: 33. https://doi.org/10.6620/ZS.2017.56-33

Marin IN, Palatov DM (2023) Insights on the existence of ancient glacial refugee in the Northern Black/Azov Sea lowland, with the description of the first stygobiotic microcrustacean species of the genus *Niphargus* Schiödte, 1849 from the Mouth of the Don River. Diversity 15(5): 682. https://doi.org/10.3390/d15050682

Morhun H, Son MO, Rewicz T, Kazanavičiūtė E, Copilas-Ciocianu D (2022) The first records of *Niphargus hrabei* and *N. potamophilus* in Ukraine and Bulgaria significantly enlarge the ranges of these species. The European Zoological Journal 89(1): 1191–1200. https://doi.org/10.1080/24750263.2022.2126534

Ozkahya P, Camur-Elipek B (2015) A study on determining of macroinvertebrate biodiversity in water wells with stygobiont species findings. Biologija (Vilnius, Lithuania) 61(3–4): 167–172. https://doi.org/10.6001/biologija.v61i3-4.3209

Posada D (2008) jModelTest: Phylogenetic model averaging. Molecular Biology and Evolution 25: 1253–1256. https://doi.org/10.1093/molbev/msn083

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics (Oxford, England) 19(12): 1572–1574. https://doi.org/10.1093/bioinformatics/btg180

Stoch F, Christian E, Flot J-F (2020) Molecular taxonomy, phylogeny and biogeography of the *Niphargus tatrensis* species complex (Amphipoda, Niphargidae) in Austria. Organisms, Diversity & Evolution 20(4): 701–722. https://doi.org/10.1007/s13127-020-00462-z

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular evolutionary genetics analysis using maximum l2015ikelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution 28(10): 2731–2739. https://doi.org/10.1093/molbev/msr121

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice 22: 4673–4680. https://doi.org/10.1093/nar/22.22.4673

Väinölä R, Witt J, Grabowski M, Bradbury JH, Jazdzewski K, Sket B (2008) Global diversity of amphipods (Amphipoda; Crustacea) in freshwater. Freshwater Animal Diversity Assessment, 241–255. https://doi.org/10.1007/978-1-4020-8259-7_27

Verovnik R, Sket B, Trontelj P (2005) The colonization of Europe by the freshwater crustacean *Asellus aquaticus* (Crustacea: Isopoda) proceeded from ancient refugia and was directed by habitat connectivity. Molecular Ecology 14(14): 4355–4369. https://doi.org/10.1111/j.1365-294X.2005.02745.x

Weber D, Weigand AM (2023) Groundwater amphipods of the hyporheic interstitial: A case study from Luxembourg and The Greater Region. Diversity 15(3): 411. https://doi.org/10.3390/d15030411

Zakšek V, Sket B, Trontelj P (2007) Phylogeny of the cave shrimp Troglocaris: Evidence of a young connection between Balkans and Caucasus. Molecular Phylogenetics and Evolution 42(1): 223–235. https://doi.org/10.1016/j.ympev.2006.07.009